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Occurrence of Chlorogenic Acid as the Substrate of the Enzymatic Browning Reaction in Head Lettuce, *Lactuca sativa* L.

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Summary

A spectrophotometric investigation was undertaken to elucidate the natural substrate of enzymatic browning in head lettuce (*Lactuca sativa* L.).

The lettuce extract was oxidized by its polyphenol oxidase (EC 1.10.3.1,) and a remarkable browing of the reaction mixture was observed. The difference spectra of the lettuce extract during the reaction showed a negative peak at 325 nm and positive ones at 260 and 380 nm. The spectral profiles of the difference spectra were similar to those found in the enzymatic oxidation of chlorogenic acid (5-caffeoyl quinic acid, Chl).

The lettuce extract was separated into two phenolic fractions (F-I and F-II) by DEAE-Toyopearl column chromatography and the enzymatic browning was limited in F-II. The spectral profiles and the color reactions on paperchromatogram of F-II almost coincided with those of Chl or its analogues.

These results suggest that a Chl analogue is presented in the head lettuce as substrate of the browning reaction.

Key words; head lettuce, *Lactuca sativa* L., enzymatic browning, chlorogenic acid, absorption spectra, difference spectra

1. Introduction

Recentry, there has arisen a demand for lettuce (*Lactuca sativa* L.) in convinient form such as shredded lettuce. The main disadvantage of the product is the browning reaction found at the cutend of the plant, because the browning decreases the quality of it. This undesirable browning seems to be caused by the same enzymatic reaction as those which found in many fruit and vegetables[1-6].

In previous paper[7], we reported purification and some properties of lettuce polyphenol oxidase (EC 1.10.3.1, PPO). However, the substrate of the browning reaction occurred in lettuce had not yet been studied. In present work, the spectrophotometric research has done to make clear the substrate in the plant.

2. Material and Methods

2.1. Material

The head lettuce used was purchased in a local market in Saga city.

2.2. Preparation of PPO

The enzyme was prepared by the same method as described in previous paper[7]. Crude enzyme (after ammonium sulfate fractionation) was used in this work.

2.3. Preparation of lettuce extract

Five hundred gram of sliced fresh lettuce was boiled for 10 min in 1000 ml of 95% ethanol and blended using an electric mixer. The resulting slurry was boiled under reflex for 1 hr and filtered through a Toyo No. 2 filter paper. The residue on the paper was extracted twice with 250 ml of 70% ethanol and filtered. All three filtrates were combined, transferred to a rotary evaporater, concentrated in vacuo to about 30 ml and diluted with water to 200 ml.

2.4. Measurement of difference spectra

Control: A 0.5 ml of prepared PPO was added to a mixture of 0.5 ml of 5 mM known polyphenols or lettuce extract, 4 ml of 0.1 M citrate-0.2 M sodium phosphate buffer (McIlvaine buffer, pH 4.5) and 4 ml of 4% metaphosphoric acid (HPO_3). The enzyme was inactivated in the mixture by HPO_3. The mixture diluted to 1/5 strength with 2% HPO_3 was used as control. Test: A 0.5 ml of the prepared PPO was added to a mixture of 0.5 ml of the substrate solutions described above and 4 ml of same McIlvaine buffer. After incubation for 5 or 10 min at 30 °C, 0.5 ml of this reaction mixture was diluted with 4.5 ml of 2% HPO_3 to stop the reaction.

Absorption spectra and difference spectra between control and test solutions were measured by a Hitachi model 557 spectrophotometer.

2.5. DEAE-Toyopearl column chromatography of lettuce extract

Five ml of the lettuce extract were applied to a DEAE-Toyopearl column (11 x 25 mm) equilibrated with 0.01 M phosphate buffer (pH 7), and then the column was eluted with the same buffer. Polyphenols adsorbed on the column were eluted with 0.1 N HCl. The adsorbed fractions were pooled and dried in vacuo, and dissolved with 5 ml of water.

2.6. Assay for browning reaction of DEAE-Toyopearl fractions

To a mixture of 1.0 ml of each DEAE-Toyopearl fraction and 3.5 ml of McIlvaine buffer (the mixture was adjusted to pH 4.5), 0.5 ml of the prepared PPO solution was added and incubated at 30 °C. Time course of the reaction was measured by a Ubest-30 spectrophotometer (Japan spectroscopic Co. Tokyo).

2.7. Paper chromatography of DEAE-Toyopearl fraction

Two dimensional chromatography on Toyo No. 51B filter paper (20 × 20 cm) was applied to separate the polyphenolic constituents of DEAE-Toyopearl fraction. Solvent systems and developers are described in Table 1.

3. Results and Discussion

3.1. Spectrophotometric research on the substrate of the browning reaction in lettuce extract

The lettuce extract was oxidized by its PPO and a remarkable browning was observed in the reaction mixture. The absorption spectra and difference spectra of the mixture varied with the oxidative reaction. Figure 1 shows absorption spectra and difference spectra of the lettuce extract after the addition of its PPO. As oxidative reaction proceeded, absorbance of the reaction mixture decreased in the ranges of 280 to 370 nm and increased in both ranges of 220 to 280 nm and 370 to 500 nm. In the difference spectra, a negative peak was found at 325 nm and positive ones at 260 and 380 nm.

We already reported that the lettuce PPO strongly oxidized Chl and (−)-epicatechin (Epi)[7]. Therefore, the spectral profiles of these polyphenols were determined.

Figure 2 shows the spectral profiles of Chl-PPO system. During Chl oxidation, a negative peak was found at 325 nm and positive ones at 260 and 375 nm in difference spectra. The spectral profiles of Chl-PPO system were almost coincided with those of lettuce extract-PPO system as shown in Fig. 1.

On the other hand, different absorption spectra were found in the oxidative reaction in Epi-PPO system: the absorbance increased in the whole ranges of 220 to 500 nm, and positive peaks were found at 245 and 380 nm in difference spectra (Fig. 3). In pyrocatechol -PPO and DL-dopa-PPO systems, absorbances of the reaction mixtures increased in the whole ranges of 220 to 500 nm as well as in Epi-PPO system.

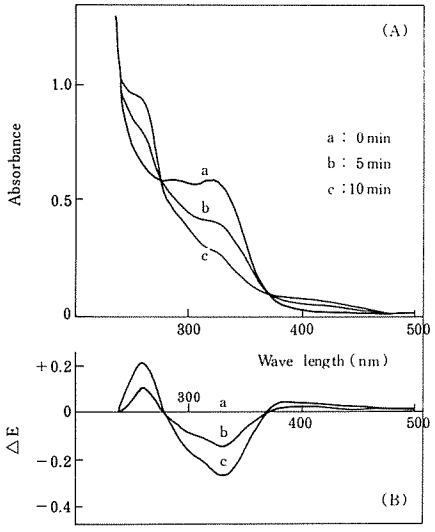

Fig. 1 Absorption spectra (A) and difference spectra (B) of lettuce extract during browning reaction by its PPO.

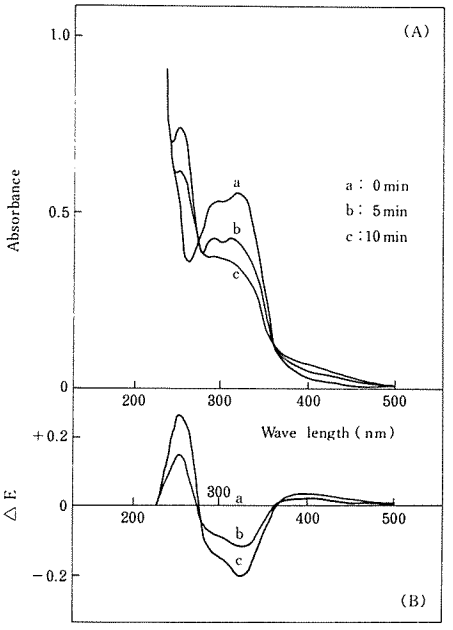

Fig. 2 Absorption spectra (A) and difference spectra (B) of chlorogenic acid during browning reaction by lettuce PPO.

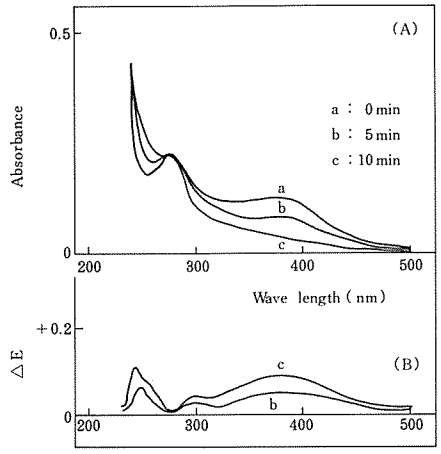

Fig. 3 Absorption spectra (A) and difference spectra (B) of (−)-epicatechin during browning reaction by lettuce PPO.

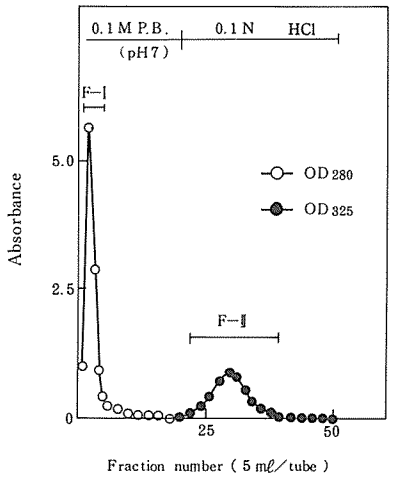

Fig. 4 Elution pattern of lettuce extract on DEAE-Toyopearl 650M.

These results suggest that oxidation of Chl or its analogues mainly cause enzymatic browning in lettuce. Similar spectral profiles were reported in Satsuma mandarin[8, 9] and Japanese pear fruits[10] during the enzymatic oxidation of various polyphenols by their PPO.

3. 2. Separation and browning reaction of polyphenols in the lettuce extract

Elution pattern of the lettuce extract on DEAE-Toyopearl 650M is shown in Fig. 4. The extract was separated into two fractions by the column: the fraction passed through the column (F-I) and the adsorbed one eluted with 0.1 N HCl (F-II).

Figure 5 shows the browning reaction of the two fractions by lettuce PPO. Browning was found only when F-II was used as sub-

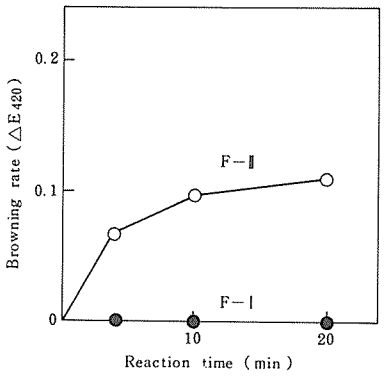

Fig. 5 Browning reactions of DEAE-Toyopearl fractions (F-I and F-II) by lettuce PPO.

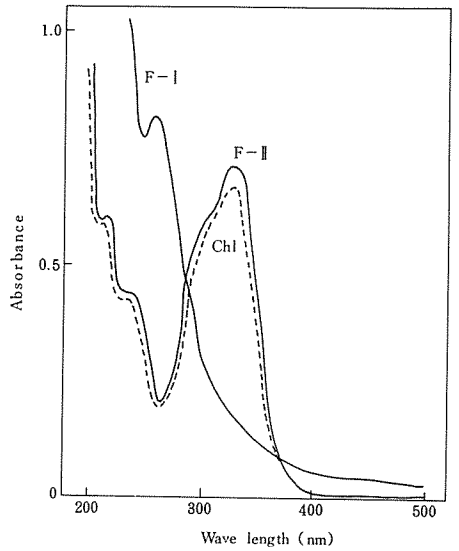

Fig. 6 Absorption spectra of F-I, F-II and chlorogenic acid.

Table 1 Paper chromatography of F-II obtained by DEAE-Toyopearl chromatography

	Rf value		Color reactions			
	BAW (4:1:2)	AW (2:98)	Fluorescence under UV-light	Fluorescence under (+NH_3)	Diazotized p-nitro aniline	$FeCl_3$-$K_3Fe(CN)_6$
Spot 1 (S_1)	0.73	0.65	Blue	Green	Brown	Blue
Cholorogenic acid	0.65	0.56	Blue	Green	Brown	Blue
Caffeic acid	0.85	0.25	Blue	Blue	Brown	Blue
Pyrocatechol	0.93	0.74	—	—	Brown	Blue
DL-DOPA	0.36	0.82	—	—	Yellowish orange	Blue
(−)-epicatechin	0.61	0.40	—	—	Yellow	Blue
Pyrogallol	0.75	0.68	—	—	Yellow	Blue
Gallic acid	0.62	0.36	—	—	Brown	Blue

B; n-butanol, A; acetic acid, W; water, —; colorless

strate.

Absorption spectra of F-I, F-II and Chl are shown in Fig. 6. Absorption peak of F-I was found at 275 nm and the peaks of F-II were at 215, 240 and 325 nm. The absorption spectrum of F-II coincided with that of Chl. Polyphenols were similarly separated using DEAE-ion exchangers in the extracts of Satsuma mandarin[11, 12] and Japanese pear[13], and the main polyphenols in F-II of these fruits extracts were regarded as Chl analogue.

Table 1 shows the chromatogram of F-II on Toyo No. 51B filter paper. One spot (spot S) was detected on the paper by fluorescence and other color reactions. Although *Rf* values of the spot S slightly differed from those of Chl, color reactions of the spot S coincided well with those of Chl. Therefore, the spot S seems to be a Chl analogue, but it is not identified.

All these results indicate that a Chl analogue is distributed in the lettuce and the analogue is oxidized by the PPO to cause browning reaction at a cutend of lettuce.

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結球レタス（*Lactuca sativa* L.）中の酵素的褐変反応基質としてのクロロゲン酸の存在

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摘　　要

結球レタスに存在する酵素的褐変反応の主要基質について主として反応液の吸収スペクトルの面から追究した．

レタス抽出液はそのポリフェノール酸化酵素により強く酸化され，その反応液は著しく変色した．その際，レタス抽出液の差スペクトルは325nm に負のピークを，260及び380nm に正のピークを示した．このような差スペクトルの変化の特徴はクロロゲン酸の酵素的酸化時にみられるものと類似した．

レタス抽出液は DEAE-トヨパールクロマトグラフィーにより２つのフェノール画分（F-I及びF-II）に分画されたが，このうちF-IIのみに酵素的褐変がみられた．また，F-IIの吸収スペクトルやペーパークロマトグラム上の呈色反応等はクロロゲン酸のそれらとほぼ一致した．

これらの結果はレタス抽出液中の褐変反応の基質として一種のクロロゲン酸同族体が存在することを示唆している．